Potential Application of *Chryseomicrobium amylolyticum* against Phytotoxicity Due To Hexavalent Chromium.

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Abstract: Hexavalent Chromium (Cr) is considered as a severe pollutant due to its non-biodegradability and thus it causes bio-accumulation resulting in phytotoxicity. In the present study, Cr resistant *Chryseomicrobium amylolyticum* JC16 was obtained from chromium containing electroplating effluent sample. Minimum inhibition concentration of chromium for *C. amylolyticum* was determined as 1300µg/ml. The reduction assay showed that this isolate was able to reduce Cr completely in 48hrs of the incubation period. Optimization study revealed that maximum chromium reduction efficiency could be achieved at temperature 30°C, neutral pH and at initial Cr concentration of 100µg/ml. Further, this isolate was used for treatment for 100µg/ml Cr spiked river water. The treated effluent water was studied for the effects of Cr on plant *Vigna radiata*, in comparison with untreated effluent. Due to the treatment of effluent, seed germination increased up to 70% compared to only 30% in untreated effluent. The different parameters including root, shoot and seedling length were studied. The results were favorable with treated effluent than untreated. The Vigour index and Tolerance index was more in treated effluent compared with untreated effluent. Hence it was concluded that *C. amylolyticum* can be a tool of bioremediation against Cr phytotoxicity.

Key Words*- Electroplating Effluent, Reduction assay, Vigour index, Tolerance index, *Vigna radiata.

1. INTRODUCTION

Chromium exists in a number of oxidation states from Cr (III) to Cr (VI). The most stable forms are Cr (VI) and Cr (III). Chromium is a significant metal due to its high corrosion resistant ability and hardness. Hexavalent chromium is extensively used in the number of industries which includes electroplating of stainless steel, leather tanning, fabrication, dying, cement, wood preservation, ceramics, therefore chromium act as the most frequent pollutant in a wide variety of industrial discharges. (Mistry K. et al., 2010). Mostly Chromium is discharged in industrial effluents without any treatment. Hexavalent chromium is a toxic and carcinogenic metal, causes pollution of water bodies, soil, and also causes health hazards. According to the World Health Organization [WHO] drinking water guidelines, the maximum permissible limit for hexavalent chromium Cr (VI) and total chromium are 0.05 and 2 mg/lit respectively.

Cr (VI) is highly mutagenic because unlike other metals it directly reacts with DNA forming protein and DNA–DNA cross-links. Zinc chromate is the strongest carcinogen of the chromate used in industries. A soluble compound like chromic acid is much weaker carcinogens. The accumulated chromium in the soil can also cause severe and long term toxic effects on the soil ecosystem and also influence plant metabolism, growth and seed germination (Atta M.I. et al., 2013). Due to deprived translocation of Cr; it gets accumulated 100 fold higher in roots than the shoot, followed by leaves and then fruit. It can absorb both as Cr (III) or Cr (VI), but there is no specificity for Cr uptake. However, plants uptake Cr along with water. Members of family *Brassicaceae* are sulfur loving plants and they have been establishing to accumulate the maximum amount of Cr due to the Cr translocation in the plants via sulfur uptake mechanism. Cr (III) is taken up passively by simple diffusion whereas Cr (VI) is transported actively by sulfate carriers. Cr is a nonessential element for plants. The solubility of Cr (VI) in water is a hazard for biota (Singh H. et al., 2013). Terrestrial as well as aquatic plants have been affected by Cr (VI). (Chandra and Kulshreshtha, 2004). Cr (VI) reduced the number of palisade and spongy parenchyma cells in leaves, induced clotted depositions in the vascular bundles of the stems and root, enhanced the number of vacuoles in the wall of xylem and phloem. Cr (VI) has been reported to cause complete obliteration of cortical tissue in the root. In the plant Cr (VI) induced oxidative stress, reduces growth and yield, stunted plant growth and finally plant death is also instigated by Cr (VI) toxicity.

Chemical method is often obtainable for the abolition of chromium in majority from industrial effluent. These methods have certain disadvantages includes high cost, low efficiency, and generation of toxic sludge or other wastes. So, Bioremediation may be a better substitute to chemical methods. Microorganisms can consume these compounds for their growth and energy needs for their metabolic pathway. Therefore these organisms can breakdown pollutant molecules concurrently and solve the environmental problem (Mistry K. et al., 2010).

The mechanisms by which these microorganisms can reduce Cr (VI) are variable and depend on a variety of bacterial species. A chromate resistant bacteria isolates have been reported and the mechanism of resistance to this ion may be encoded either by plasmids or by the chromosomal gene. In *Pseudomonas aeruginosa,* Chr A transporter protein causes efflux of cytoplasmic chromate (Cervantes C. and Campos Garcia J., 2007). On the other hand resistance systems restricted to a bacterial chromosome are generally specific or unspecific Cr (VI) reduction, free radical detoxifying activities repairing of DNA damage. The enzyme chromate reductase causes reduction of Cr (VI) to Cr (III) in various species. (Ramirez Diaz M. I., et al., 2011).

In this context, the present investigation was aimed to study the bioremediation efficiency of Chromium resistant isolate to reduce the phytotoxicity of chromium.

2. MATERIALS AND METHODS:

2.1 Sample collection:

Effluent samples were collected from the electroplating industry located in Nanded MIDC, in glass sampling bottles, which were washed with 8M HNO_3 solution and then sterilized in an autoclave at 121°C, at 15 lbs for 15 minutes. All samples were transported aseptically in the icebox to the laboratory immediately and stored at 4°C for further analysis.

2.2 Measurement of Different Heavy Metals in Samples:

Different types of heavy metals are involved in the various electroplating processes. Hence by using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) determination of such heavy metals were carried out. (BhishnurkarP.G. et al., 2015; Jusufi K. et al., 2016)

2.3 Isolation and identification of chromium resistant bacteria:

The sample was serially diluted up to 10^{-6} in sterile saline. 1 ml of each dilution was spread on nutrient agar plates which were amended with 200µg/ml of Cr (VI) and plates were incubated at 30 for 24 hrs. After the incubation period, the plates were observed for the growth of Cr (VI) resistant colonies. Morphologically different colonies were selected and purified by re-streaking on nutrient agar plates. The isolated colonies were maintained on nutrient agar slants. Further identification was done on the basis of biochemical characteristics and 16s r RNA sequencing.

2.4 Determination of MIC of chromium resistant isolates:

MIC is the lowest concentration of an antimicrobial compound that inhibits the visible growth of microorganisms after overnight incubation. The MIC of chromate for each isolate was determined by the agar dilution method. Different chromium concentration of 100, 200 up to 1500µg/ml dilution were prepared in sterile molten nutrient agar. These chromium supplemented plates were spread with 0.1 ml of overnight culture growth of isolate and incubated at 30 for 24 hrs. The concentration at which, inhibition of growth was observed, recorded as MIC.

2.5 Cr (VI) reduction Assay:

The isolate was cultured in nutrient broth for 24hrs. Reduction of Cr (VI) was determined by inoculating 24 hrs old culture of the isolates in 100ml nutrient broth amended with 100µg/ml Cr (VI) and incubated at 30 with agitation 100rpm. Reduction of chromium was measured after each 24 hrs interval. The sample was centrifuged at 6000rpm for 10 min. and the supernatant was analyzed for remaining Cr (VI) by Diphenyl Carbazide method (DPC) (APHA). Cr (VI) reduced by isolates was calculated by using formula as follows: (Chen Y., et al., 2018)

% Cr (VI) Reduction =

where,

$\mathbf{I_{cr}}$ – Initial Cr (VI) concentration (µg/ml)
$\mathbf{F_{cr}}$ – Final Cr (VI) concentration (µg/ml)

2.6 Optimization of different parameters for Cr (VI) Reduction:

Temperature, pH and initial chromium concentration were considered as important parameters in the optimization experiment. Chromium reduction efficiencies of the isolate were determined at various temperature (10, 20, 30, 40 and 50 , pH (4, 5, 6, 7 and 8) and initial Cr (VI) concentrations (100, 200 up to 500µg/ml). The respective pH was adjusted with 0.1 N NaOH and 0.1 N HCl solutions.

2.7 Treatment of effluent for bio removal of chromium:

The effluent sample was prepared by adding 100µg/ml of Cr (VI) in river water. This effluent was inoculated with 4% inoculum (24 hrs old culture of isolate) incubated at 30 for 24 hrs, at 100rpm and considered as treated sample. Samples were collected initially and after 24hrs of the incubation period and centrifuged. The amount of chromium was determined spectrophotometrically by using the DPC method. The effluent sample without inoculation was considered as untreated effluent. These treated and untreated effluents samples were filtered through filter paper and sterilized at 121°C for 15min, 15lbs in an autoclave. These effluent samples were used for further study.

2.8 Effect of treated and untreated effluent on seed germination of the *V. radiata*:

Cr (VI) bio removal efficiency of the resistant isolate was determined by examining the effect of treated and untreated effluents on the seed germination of *V. radiata*, under the laboratory conditions. For this study, local farm soil was collected and air-dried for 2-3 days. The pots were filled with an equal amount of soil. These pots were rehydrated with treated, untreated effluents and control pot with distilled water. The seeds of *V. radiata* were purchased from a local market. Surface sterilization of seeds was carried out by washing with 0.1% $HgCl_2$ solution and then with deionized distilled water. (Amin H., et al., 2013). Twenty seeds of *V. radiata* were sown uniformly in pots. The treated and untreated effluent samples were used for seed irrigation regularly. Similarly, the control pot seeds were irrigated with distilled water. Germination and growth of seedlings were measured for 14 days. The seedling tests were conducted according to the Seedling Evaluation Handbook, Association of Official Seed Analysts (AOSA, 1981), which included seed germination, germination time, root length, shoot length, seedling length, Seedling Vigour index, Tolerance index and percentage of phytotoxicity. (Masuthi D. et al., 2015; Murtaza S. et al., 2017; Nagarajan N. et al., 2012). The Seedling Vigour Index (S.V.I.), Tolerance Index (T.I.) and Percentage of Phytotoxicity (P.P.) are calculated are as follows.

Seedling Vigour Index.

S.V.I. = [Seedling length (in cm) × Germination percentage]

Tolerance Index.

T.I. = $\frac{\text{Mean le}}{\text{Mean}}$

Percentage of phytotoxicity

P.P. = $\frac{\text{Radical lengt}}{} \times 100$

3. RESULTS AND DISCUSSION:

3.1 Determination of Heavy Metals present in Sample:

In the collected effluent sample, heavy metals including Cr, Cu, and Zn were present. Other heavy metals Cd, Co, Ni, and Pb were not detected (Table 1).

Table 1: Concentration of heavy metals in effluent samples (in µg/ml)

HeavyMetals	Cd	Co	Cr	Cu	Ni	Pb	Zn
Concentrations	ND	ND	5.889	0.021	ND	ND	0.033

ND means < 0.01 µg/ml.

3.2 Isolation, identification, and MIC determination:

On the basis of distinct morphological characteristics, nineteen well-defined colonies were selected from Cr (VI) amended plates. All Nineteen Cr resistant isolates were determined for MIC of chromium, which showed range from 200-1300µg/ml. Isolates SICr03, showed higher MIC value i.e. 1300µg/ml, which was selected for further study. Morphological and biochemical tests for isolates SICr03 showed Gram +ve rods, non spore-forming, catalase positive, and H_2S negative. Identification of isolate SICr03 by 16S- r RNA sequencing showed 99% similarity with *Chryseomicrobium amylolyticum* JC16. Xiao W. et al., (2017) reported that, isolates *Bacillus sp*. FY1 and *Arthrobacter* sp. WZ2 were tolerant to 1000µg/ml Cr (VI).

3.3 Chromium reduction assay:

Isolate *C. amylolyticum* could reduce 100µg/ml of Cr (VI) up to 51% and 100% in 24 and 48 hrs of the incubation period. (Fig.1). Elangovan R. et al., (2006) reported that chromate resistant *Bacillus* sp. showed 80% Cr (VI) reduction after 64hrs of the incubation period, with 40µg/ml, but with 80µg/ml, maximum Cr (VI) reduction was 50%.

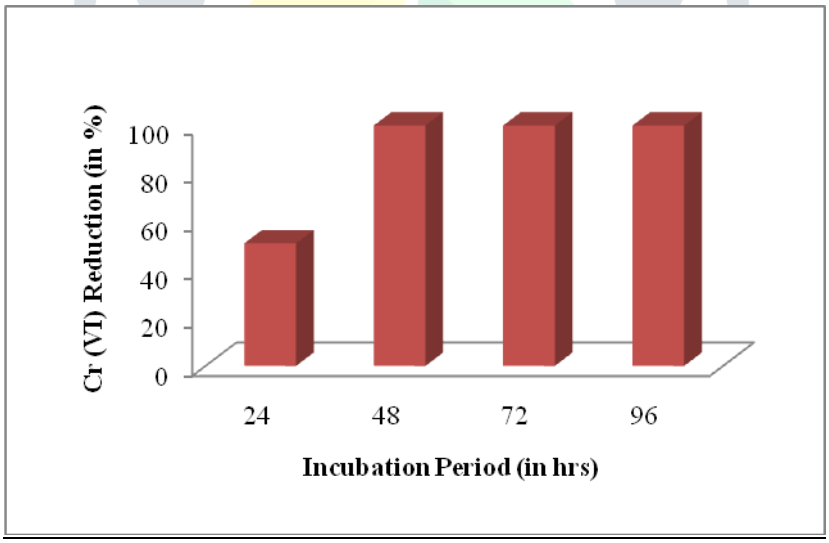

Fig 1: Chromium reduction assay

3.4 Optimization of different parameters for Cr Reduction:

The effect of temperature on Cr (VI) reduction by isolates was depicted in fig.2, isolates *C. amylolyticum* showed the complete reduction of Cr (VI) at temperature 30°C in nutrient broth containing 100µg/ml of Cr. Effect of different pH on percentage Cr (VI) reduction was given in fig. 3. At pH 7 isolates *C. amylolyticum* was able to reduce Cr (VI) maximum with an efficiency of 100%. The reduction efficiency of these isolates was decreased in acidic and basic pH. Silva B., et al., (2009) revealed that maximum Cr removal efficiency (72.5%) was achieved at pH 4, after 73 days of contact time.

As shown in fig.4, isolate showed a decrease in the efficiency of Cr (VI) reduction as increase in Cr concentration. There was a reciprocal relation between initial Cr (VI) concentration and reduction efficiency. Isolate *C. amylolyticum* could show the complete reduction of 100µg/ml Cr (VI) after 48hrs of the incubation period. Megharaj M. et al., (2003) reported that, during 46

hrs of the incubation period, isolates *Arthrobacter* sp. and *Bacillus* sp. showed the complete reduction of Cr(VI) in concentration only up to 30µg/ml and 10µg/ml respectively. Further, he stated that *Arthrobacter* sp. did not show any Cr reduction at 100µg/ml.

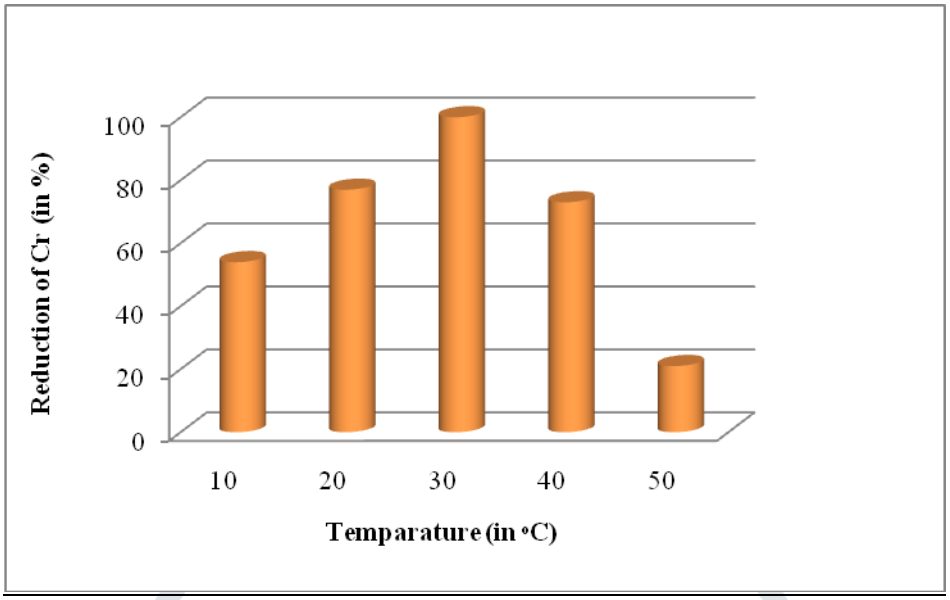

Fig 2: Effect of Temperature on Cr(VI) Reduction

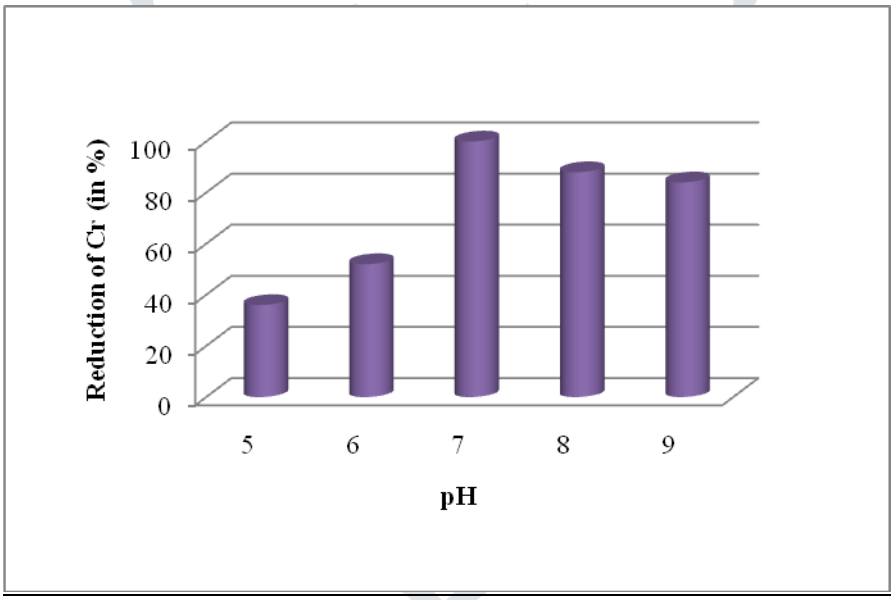

Fig 3: Effect of pH on Cr(VI) Reduction

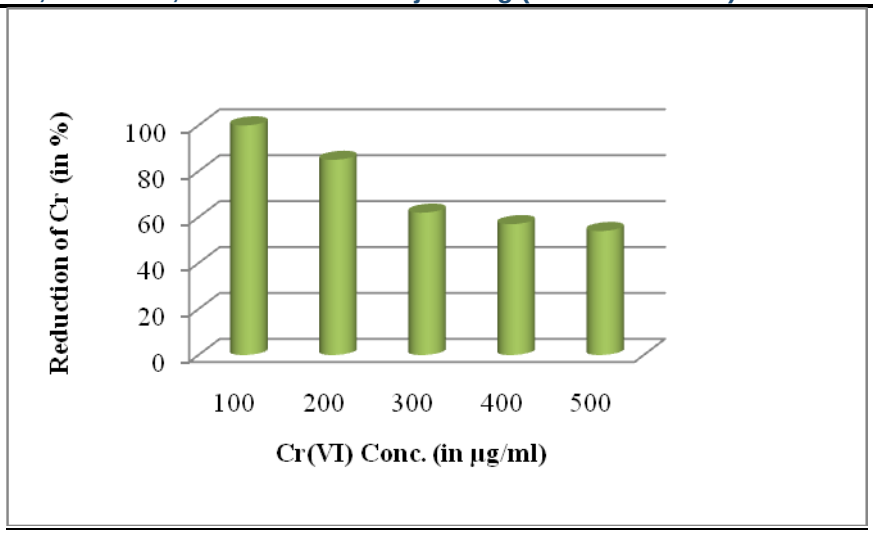

Fig 4: Effect of Initial Concentration on Cr (VI) Reduction

3.5 Treatment of effluent for bioremediation of chromium:

The *C. amylolyticum*could achieve half of the initial concentration of chromate in the effluent in 24 hrs. This treated effluent sample was used for the study of effects on growth parameters of *V. radiata* in comparison with the untreated effluent sample.

3.6 Effect of treated and untreated effluent on the growth of *V. radiata*:

70% of seeds were germinated in treated effluent while 30% of seeds were germinated in untreated effluent as compared to control (Table 2). However, as shown in Fig. 5, a significant difference in seed germination could be noticed in treated effluent and untreated effluent. Seed germination is considered as physiological activity which can be activated under enzymatic activity by water imbibitions. The Cr (VI) mainly repressed such enzymatic activities, which badly affects the seed germination. Other parameters of growth like root and shoot length were also increased in the case of seed irrigated with treated effluent in comparison with untreated effluent. Size of seedling was increased up to 9.8 cm in *V. radiata* irrigated with treated effluent and in *V. radiata* irrigated with untreated effluent seedling size was 1.0cm.

As shown in Table 3, Seedling Vigour Index was recorded low i.e. 30 in *V. radiata* irrigated with untreated effluent and was increased up to 686 after treatment of effluent. Similarly, after treatment of effluent Tolerance index of *V. radiata* was increased up to 0.725 and in case of *V. radiata* irrigated with untreated effluent Tolerance index was 0.125. The study of percentage phytotoxicity revealed that phytotoxicity caused in *V. radiata* due to untreated effluent was 87.5; this phytotoxicity was able to reduce up to 27.5 due to effluent treated with *C. amylolyticum*. Nagarajan M. and Sankar G. K., (2014) reported that, in case of Paddy plant (*Oryza sativa* L), due to effect of chromium germination percentage in control (98.0 3.2) was reduced up to (58.0 1.4) due to 100µg/ml of Cr.

Table 2: Effect of treated and untreated effluent on growth parameters of *V. radiata*

	Seed Germination Percentage	Germination Time (in days)	Mean Root Length (in cm)	Mean Shoot Length (in cm)	Seedling Length (in cm)
Control	80	5	8.0	12.5	20.5
Treated Effluent	70	7	3.8	6.0	9.8
Untreated Effluent	30	12	1.0	00	1.0

Table 3: Effect of treated and untreated effluent on Vigour Index, Tolerance index and Percentage of phytotoxicity

	Vigour Index	Tolerance Index	Percentage Phytotoxicity
Control	1,640	--	--
Treated Effluent	686	0.725	27.5
Untreated Effluent	30	0.125	87.5

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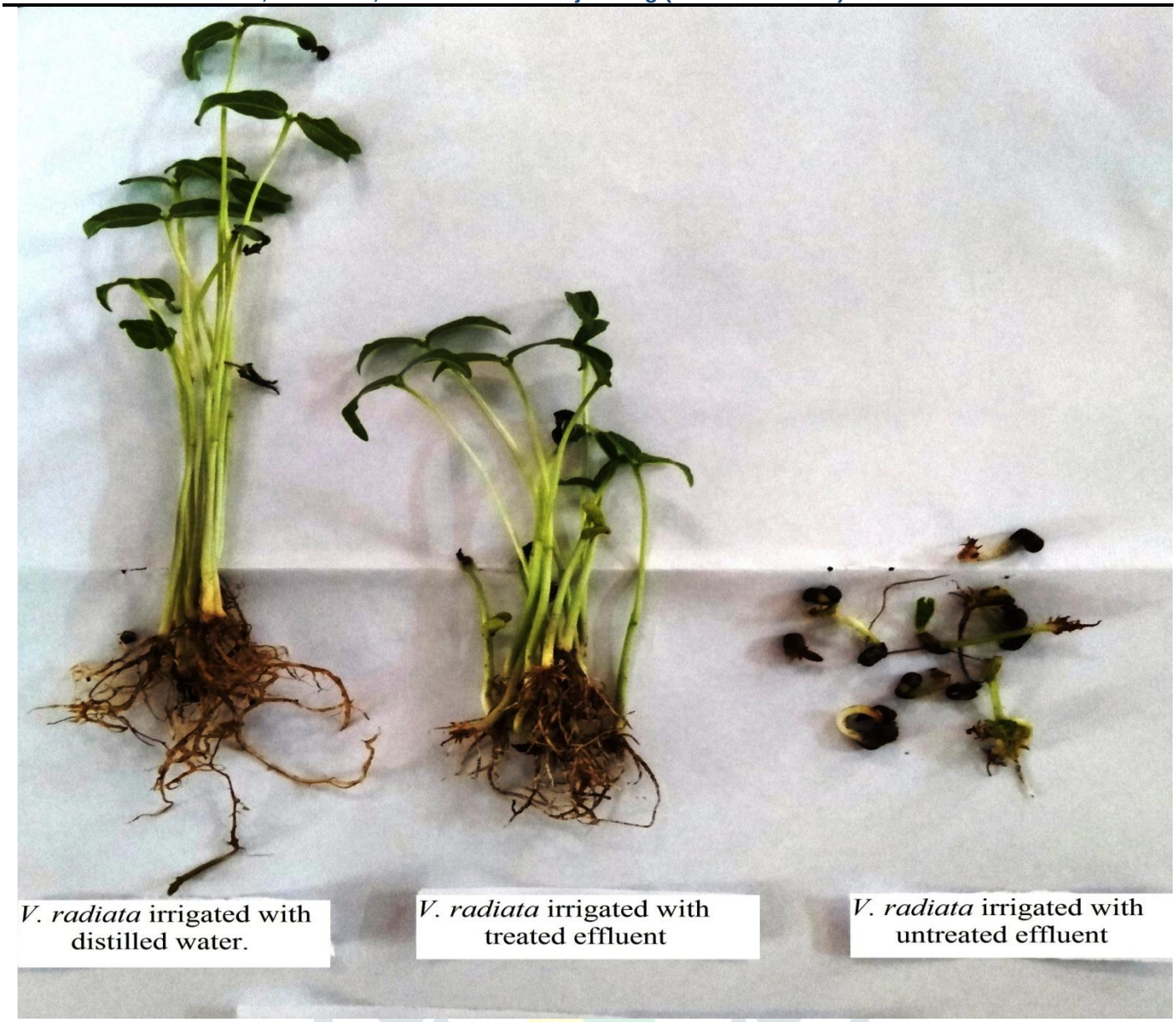

Fig 5: Effect of treated and untreated effluent on the growth of *V. radiata*

4. CONCLUSIONS:

The present investigation has examined the presence of indigenous organisms in the Cr (VI) contaminated industrial effluent. It was identified that an organism *C. amylolyticum* showed high resistance to Cr (VI) i.e. 1300µg/ml and has significantly reduced toxic Cr (VI) 51% to its nontoxic Cr (III) form in the effluent. Hence *C. amylolyticum* was used for the treatment of effluent water; the result showed that after application of treated effluent, normal growth of plant occurred and their time period for seed germination was reduced in comparison with the untreated sample. Seed germination, germination time, seedling length (root and shoot length) found to be better as compared to untreated effluent. The untreated effluent showed inhibition of seed germination and seedling growth due to chromium. It is a toxic heavy metal that induces toxicity in the plant. Due to the phytotoxic effects of untreated effluent, it is confirmed that the high concentration of hexavalent chromium may affect the seed germination and other growth factors. In effluent sample treated with *C. amylolyticum* could reduce the toxic Cr (VI) to its nontoxic Cr (III) form. This is recommended that, by treating chromium contaminated effluent may be safe for irrigation purpose. The treated effluent water may be reused for irrigation safely.

5. ACKNOWLEDGMENT:

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